A MICROPROPAGATION SYSTEM FOR *TYLOPHORA INDICA* AND EXTRACTION OF TYLOPHORINE FROM CULTURES AND *IN VITRO* RAISED PLANTS

Thesis submitted in

partial fulfillment for the award of Degree of

Master of Science in Biotechnology

By

SUMIT SHARMA

Registration No. 300901020

Under the supervision

Of

Dr. MANJU ANAND

Assistant Professor

Department of Biotechnology and Environmental Sciences

Thapar University

Patiala-147004

JUNE 2011

CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled " **A Micropropagation system for *Tylophora indica* and extraction of tylophorine from cultures and *in vitro* raised plants**" in partial fulfillment of the requirement for the award of degree of Master of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is an authentic record of my own work done during the period of six months from January 2011 to June 2011, under the guidance of **Dr. Manju Anand**, Assistant Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

Date: 6-07-2011

Place: Patiala

Sumit Sharma

SUMIT SHARMA

CERTIFICATE

This is to certify that the thesis entitled, "**A Micropropagation system for *Tylophora indica* and extraction of Tylophorine from cultures and *in vitro* raised plants**" submitted by Sumit Sharma in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, Thapar University, Patiala, is an authentic record of his own work carried out by him during the period of six months from January 2011 to June 2011, under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

Manju Anand

Dr. Manju Anand
Assistant Professor,
Supervisor.

Dr.M.S.Reddy
Head

Department of Biotechnology & Environment Sciences
Thapar University,
Patiala.

Dr. S. K. Mohapatra
Dean, Academic Affairs,
Thapar University,
Patiala.

ACKNOWLEDGEMENT

First of all, I express my deepest sense of gratitude and indebtedness to the Almighty whose blessings have enabled me to accomplish my thesis work successfully.

It is my privilege to express my sincere thanks and a sense of deep gratitude to **Dr. Manju Anand**, Assistant professor, Department of Biotechnology and Environmental Sciences, for her valuable advice, splendid supervision, and immense patience due to which this work was able to take the shape in which it has been presented. It was her valuable discussions and constant encouragement through which I have gained a lot. I owe thanks to her for all the help, painstaking efforts and deep insight into the problem and thus improving the quality of work at all the stages.

I would like to acknowledge with much appreciation **Dr. Dinesh Goyal**, Co-ordinator , S.T.E.P for providing me the facilities for carrying out extraction and HPTLC studies.

My sincere thanks are due to **Dr. M.S Reddy**, Head, DBTES for providing me the best laboratory facilities during the work.

I also wish to express deeply my gratefulness to research scholar **Harmanjit Kaur** for her support and help throughout the period of my work. I am highly thankful to all my friends especially **Amit, Ravinder, Sushant, Kamal, and Veni** for making moments spent here to be cherished for lifetime and laboratory staff of DBTES for their support during my project.

The whole contribution for my achievements goes to my parents and my brother who always stood by me during thick and thin and providing me the moral support and resources to finish my work. Their unwavering faith in me has been a source of constant inspiration for me.

Dated: 6-07-2011

Place: Patiala

Sumit Sharma

(SUMIT SHARMA)

CONTENTS

Chapters **Page No.**

ABBREVIATIONS

Å	Angstrom
BAP	Benzylaminopurine.
BMS	Basal Murashige and Skoog's medium
MS	Murashige and Skoog's medium
^{0}C	Degree Celcius
NAA	Napthalene acetic acid
2,4-D	2,4-dichlorophenoxy acetic acid
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
2-ip	2-isopentyl adenine
Kn	Kinetin
Zn	Zeatin
AS	Adenine sulphate
TDZ	Thidiazuron
NK	MS+ NAA (29.4μM) + Kn (4.65μM)
ppm	part per million
μM	micro molar
W	watt
R_f	Retention factor
nm	nanometer

Abstract

Tylophora indica,(Burm.f.) Merill (Family Asclepiadaceae) is an important endangered medicinal plant which is used as a folk remedy for the treatment of a number of diseases and ailments. The roots and leaves of this plant contain pharmacologically active alkaloids tylophorine, tylophorinine and anticancerous tylophorinidine. Due to the lack of adequate propagation efforts and over exploitation of natural wild populations, *Tylophora indica* is threatened with extinction. We present an efficient and reproducible protocol for the mass propagation of this plant under *in vitro* conditions and extraction of tylophorine-the major secondary metabolite from callus, suspension cultures and *in vitro* regenerated plants.

Leaf explants were excised from an elite field grown mature plant and thereafter planted on variously supplemented Murashige and Skoog's medium for the *de novo* adventitious shoot formation and callus induction. *Tylophora* exhibited high degree of propensity of *de novo* shoot proliferation directly from leaf segments on MS medium supplemented with BAP (4.4µM - 22µM) either alone or in combination with adenine sulphate (1.35µM). Nodular meristemoids differentiated from the cut ends of leaf lamina after 10-12 days of culturing and covered the whole surface of leaf explant within 3-4 weeks. Eventually, these meristemoids developed into green leafy shoots. Initially, fewer shoots were formed but number increased further to 50-60 shoots per flask on subsequent subculturing in about 85% of the cultures. Regenerated shoots when 4-5cm in length were separated and subjected to rooting on root inducing media. Microshoots cultured on basal MS medium resulted in the formation of long, healthy roots within 10-12 days and complete plantlets were formed. Plantlets were then transferred to moist cotton jars covered with polybags for initial acclimatization and were kept in growth room for 10-12 days. They were then transferred to the potting mixture of soil: vermicompost (1:1) and plants with newly formed leaves were shifted to green house for 2 weeks and eventually established in soil with 90% survival rate.

Callus formation occurred from leaf explants when inoculated on different combinations of auxins and cytokinins. Murashige & Skoog's medium supplimented with NAA (29.4µM) and Kn (4.65µM), hereby designated as NK medium turned out to be optimal for the initiation and sustained growth of calli. Leaf explants also showed callus induction on MS+ 2, 4-D (38.96µM) + Kn (4.65µM) but growth of callus was slow.

Extraction of major secondary metabolite - tylophorine was carried out from the dried leaf powder of *in vitro* raised field established plants (2 years old), dried callus (3-4 months old) and dried suspension cultures raised on liquid NK medium.

Extraction was carried out with chloroform using soxhlet apparatus followed by extraction with ethyl acetate. Thin layer chromatography was performed in the solvent system of different combinations of toluene: ethyl acetate and diethyl amine but best results were observed on 7:2:1 combination. For further fine purification of tylophorine, High Performance thin layer chromatography (HPTLC) was performed using the same solvent system as mobile phase. Three tracks 1, 2, 3 were obtained corresponding to samples of dried leaf powder, callus, and suspension powder respectively. Peak 7, peak 8 and peak 7 of track 1, 2 and 3 respectively showed R_f values comparable with the standard which confirmed the presence of tylophorine in the given samples.

Introduction

Plants, since time immemorial, have been used globally as a valuable and safe natural source of medicine for basic preventive and curative healthcare. Plants synthesize and accumulate a variety of compounds which provide protection to the plant and are useful to the humans as medicinal agents.

Herbal medicine is the oldest form of healthcare known to mankind and herbs have been used by all the cultures throughout the history. Much of the medicinal use of plants seems to have been developed through observations of wild plants and by trial and error. As time went on, each tribe added the medicinal power of herbs in their area of its knowledge base. The knowledge of medicinal plants has been accumulated in the course of many centuries based on different medicinal systems such as Ayurveda, Chinese medicine, Siddha and the Japanese Kampo. Over 9000 plant species have known medicinal applications in various cultures and countries (Farnsworth and Soejarto, 1991).

The World Health Organisation (W.H.O.) estimates that 4.3 billion people or 80% of world's population, primarily those of developing countries, use herbal medicine for their healthcare needs (Gurib-Fakim, 2006). In India, it is reported that the traditional healers use 2500 plant species, out of which 100 species of plants serve as regular source of medicine (Pei, 2001). Medicinal plants form the resource base for rapidly growing pharmaceutical industry. Industrialized societies are involved in the extraction of bioactive constituents from medicinal plants and use them directly or indirectly as new drugs. As a result, there is a global resurgence in the trade of herbal medicines. The Indian systems of medicine particularly Ayurveda, Siddha, Unani and Homeopathy largely use plant based materials, minerals and metals etc. In India approximately 1700 plant species are used in Ayurveda, 500 for Siddha, 400 for Unani and 300 for Amchi systems of medicine with substantial overlap of common plants among these systems**.**

Global view of medicinal plants

Global market for medicinal plants is estimated around US $14 billion per year and is expected to cross US $5 trillion in 2050. In India, the medicinal plant related trade is estimated to be approximately US $ 1 billion per year. The global market for medicinal plants is very large. However, the real significance of medicinal plant sector has begun to be realized only during the last few decades. It has been estimated that in the country like United States, one in four prescriptions filled, are either a synthesized form or derived from plant materials (Srivastava et al., 1995). According to the International Trade Centre, as far back as 1967, the total value of imports of starting materials of plant origin for the pharmaceutical and cosmetics industry was of the order of USD 52.9 million. From this amount, the total values grew to USD 71.2 million in 1971, and then showed a steady annual growth rate of approximately 5-7% through to the mid-1980s (Atisso, 1983). Interest of world's developed countries had waned during the period of late 1960s to the early 1980s for the use of natural materials because of the new possibilities of synthesization of drugs. However in mid 1980s, interest in the use of natural materials was renewed (Tempesta and King, 1994). It was from mid 1980s onwards that natural and plant based medicines made it felt in the market.

It was found that by 1990, around 2223 major companies worldwide were reportedly screening plants for new leads and more than 2000 companies were marketing herbal medicines alone in the Europe (Tewari, 1996). Medicinal plants have gained the faith during past few years in view of their lesser side effects as compared to allopathic medicine. Drugs obtained from plants are believed to be much safer and exhibit a remarkable efficacy in the treatments of various ailments (Siddique et al., 1995).

Status of medicinal plants in India

India is a rich country in terms of its biodiversity. India is among 12 most biodiverse countries of the world having 16 agro climatic zones. Although its total land area is only 2.4% of the world, it accounts for 8% of the total global diversity. There are about 45,000 plant species in India with concentrated hotspots in the regions of Eastern Himalayas, Western Ghats and Andaman and Nicobar islands. The officially documented plants with

medicinal potential are 3000 but traditional practitioners use more than 6,000. India is the largest producer of medicinal herbs and is appropriately called the “botanical garden” of the world.

Though India has rich biodiversity, about 90% of the medicinal plants used by industries are collected from the wild. Over 70% of the plant collection involves destructive harvesting because of the use of parts like root, stems, bark, wood and whole plant in the case of herbs. The growing demand is putting a heavy strain on existing resources causing a number of species to be either threatened or included in endangered category. This poses a definite threat to the genetic stock and to the biodiversity of medicinal plants. The assessments done so far for the prioritized native medicinal species have resulted in assignment of IUCN red list status to nearly 200 of India’s medicinal plant species (Ved and Kumar, 2000).

Following is the list of some highly demanded medicinal plants traded in India:

S. No.	Botanical Name	Trade Name	Part used
1	*Aconitum heteorphyllum*, Wall. ex Royle	ATIS	Tuberous roots
2	*Aconitum violoceum* (Jacq.) Stapf	MEETHA TELIA/BACHNAG	Tuberous roots
3	*Chlorophytum arundinaceum* Baker	SAFED MUSALI	Tubers
4	*Embelia ribes* Burm f.	VIDANGA/BAIBARANGA	Fruits
5	*Coptis teeta* Wall	NANURA/MISHMIBITTER	Roots

Medicinal plants and Plant Tissue Culture

In view of the growing world population, increasing anthropogenic activities and rapidly eroding natural ecosystems, the natural habitats for a number of medicinal plants are dwindling. The rising demand for the plant based drugs is creating heavy pressure on some selected high valued medicinal plant populations due to over harvesting. As a result many of these are becoming extinct. To cope with this alarming situation, the recent advances in Biotechnology especially Plant Tissue Culture have come as a boon.

Most of the medicinal plants either do not produce seeds or seeds are too small and do not germinate in soil. Thus mass propagation of disease free planting material is the general problem. Moreover, sexually propagated plants demonstrate a high degree of heterogeneity since their seed progeny are not true-to-type unless they have been derived from inbred lines. As a result, plants raised through seeds show great variations in growth, habit and yield. Likewise, majority of the medicinal plants are not amenable to vegetative propagation by cutting or grafting.

In recent years, tissue culture has emerged as a promising technique to obtain genetically pure elites rather than having indifferent populations under *in vitro* conditions. *In vitro* propagation from very small plant parts (0.2-10mm), also called micropropagation is infact the miniature version of conventional propagation which is carried out under aseptic conditions. Micropropagation holds the significant promise for true-to-type, rapid and mass multiplication under disease free conditions. Plants raised through micropropagation are:

1. Of uniform quality and produce uniformily superior seeds.
2. Disease free and show improved vigor and quality.
3. Can be produced much more rapidly and throughout the year irrespective of season.
4. Stocks of germplasm can be maintained for many years.
5. Facilitates international exchange of germplasm without inherent risk of spreading diseases and pathogens.

TECHNIQUES OF MICROPROPAGATION:

There are three main techniques which are used for plant propagation under *in vitro* conditions:

- **Enhanced axillary shoot proliferation:**
  Micropropagation through apical and axillary shoot proliferation is the most reliable method for *in vitro* mass multiplication. Cells of meristem are uniformly diploid and are least suspeptible to genetic changes. Hence, it is the most reliable technique for mass propagation since it ensures genetic stability of clones.

- ***De novo* formation of adventitious shoots:** New adventitious shoots can develop either

A) Directly from the explants like root, stem, leaf lamina, flower parts etc.

or

B) Indirectly from callus cultures obtained from these explants.
   Plants obtained through calli may not be true elites because of high incidence of polyploidy and aneuploidy associated with callus cells and plants obtained from it.

- **Somatic embryogenesis:**
  It involves the formation of bipolar somatic embryos which can develop into fully functional plants under appropriate conditions.

Stages in Micropropagation:

Micropropagation involves four definite stages. These are as follows:

Stage 0

Selection of healthy, disease free elite mother plant for culture initiation.

Stage 1

Initiation and establishment of aseptic cultures:

Main steps involved are explants isolation, surface sterilization and establishment of explants on appropriate culture medium.

Stage 2

Shoot multiplication by using defined culture medium.

Stage 3

Rooting of regenerated shoots in *in vitro* conditions:

Shoots are separated normally from clusters and transferred to the rooting medium.

Stage 4 Transfer of plantlets to natural environment (acclimatization):

Acclimatization is the adaptation of the plant when moved to a new environment. Plants produced under *in vitro* conditions are required to be acclimatized. Acclimatization imparts some tolerance to moisture stress and a shift from heterotrophic to autotrophic nutrition. During the process of hardening, plants develop cuticle and their stomata start functioning. Hardened plantlets are then transferred to the glass or polyhouse under normal environmental conditions.

Secondary Metabolite Production

Since centuries, many plant compounds have an outstanding role in medicine. These secondary metabolites or products exert a profound physiological effect on the mammalian system and thus are known as active principles of plant. Due to their large biological activities, plant secondary metabolites have been used for centuries in traditional medicine. Nowadays, they correspond to the valuable compounds such as pharmaceutics, cosmetics, fine chemicals or more recently nutraceutics.

Production of plant secondary metabolites has, for a long time, been achieved through the field cultivation of medicinal plants. However, plants originating from particular biotopes can be hard to grow outside their local ecosystems. It also happens that common plants don’t withstand large field cultures due to the pathogen sensitiveness. This has led scientists and biotechnologists to consider plant cell, tissue or organ cultures as an

alternative ways to produce the corresponding secondary metabolites (Bourgaud et al., 2001).

Plant cell culture technologies were introduced at the end of 1960s as a possible tool for both studying and producing plant secondary metabolites. Different strategies using *in vitro* systems have been extensively studied with the objective of improving the production of secondary plant compounds. Undifferentiated cell cultures have been mainly studied, but large interest has also been shown in hairy roots and other organ cultures. There are numerous reports describing the production of diverse secondary metabolites in cultures like anthocyanins, alkaloids, carotenoids, flavones, coumarins, saponins, steroidal alkaloids, sterols, tannins, terpenoids and several others. Industrial production of secondary metabolites from the plant cell and tissue cultures is currently restricted only to three products namely shikonin, ginseng saponins and berberine. For other numerous secondary products, the *in vitro* processes are yet to achieve industrial level status. Large scale culture of plant cells is now technically feasible using bioreactors but in most cases yields of secondary metabolites is too low for commercialization.

Objectives

The present investigation was carried out on an important medicinal plant – *Tylophora indica*. This important medicinal plant is rapidly disappearing and is now listed as one of the plant species in India vulnerable to extinction. The present investigation was carried out with the following main objectives**:**

- To develop a reliable protocol for the rapid and mass scale propagation of *Tylophora indica* via direct shoot induction in short duration of time and space.

- To extract major secondary metabolite- tylophorine from callus, suspension cultures and *in vitro* raised plants of *Tylophora indica.*

Review of literature

The clonal propagation of selected phenotypes is an essential step in most of the plant breeding programmes. Sexually propagated plants demonstrate a high degree of heterogeneity since their seed progeny is not true to type unless they have been derived from inbred lines. Asexual reproduction on the other hand gives rise to plants which are genetically identical to the parent plant and thus perpetuates the unique characters of the cultivar. However, majority of the plants are not amenable to vegetative propagation by cutting or grafting thus limiting multiplication of desired cultivars. The technique of "Plant Tissue Culture" has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions.

In vitro propagation also called micropropagation is infact the miniature version of conventional propagation which is carried out under aseptic conditions. The technique of micropropagation is based on the concept of totipotency as proposed by Haberlandt. Every cell of the plant body is totipotent i.e. capable of giving rise to new plant under proper nurture conditions. Micropropagation is now a well established technique commercialized globally for the rapid production of a number of commercially important plants.

Micropropagation can be achieved by any of the three approaches:

1) Enhanced axillary shoot proliferation.
2) *De novo* adventitious shoot formation.
3) Somatic embryogenesis.

De novo formation of adventitious shoots through direct organogenesis is regarded as the most reliable method for clonal propagation because it upholds genetic uniformity among the progenies. The direct organogenesis method has the advantage of omitting the callus and embryoid phases and significantly reducing the total number of stages in culture by direct formation of new shoots from the explant. New adventitious shoots can develop directly from the explants like root, stem, petiole, leaf lamina and floral parts. Many medicinal plants like *Psorelea corylifolia* (Baskaran and Jayabalan, 2010), *Embelia ribes* (Annapurna and Rathore, 2010), *Cassia angustufolia* (Siddique *et al.*, 2010), Pyrethrum

(Hedayat *et al*., 2009), *Ophirrhiza prostrate* (Beegum *et al*., 2006), *Withania somnifera* (Kulkarni *et al*., 2000) have been successfully propagated *in vitro* by adventitious shoot formation.

Chaudhari *et al.* (2004) reported the formation of organogenic nodular meristemoids from root explants of *Tylophora indica* cultured on MS medium supplemented with BAP or 2-ip. These meristemoids showed two types of organogenic response-direct shoot bud formation (in 42% explants) and somatic embryogenesis (in 39% explants).

Nema *et al.* (2007) achieved an excellent rate of shoot multiplication from leaf explants of *Tylophora indica* on MS + BAP (11µM) + IAA (0.56µM). On the other hand a rapid *in vitro* propagation system has been developed by Bera and Roy (1993) who reported formation of multiple adventitious shoot buds from the mature leaf explants of *Tylophora indica* on MS+ BAP (22µM) + adenine sulphate (1.35µM).

Medicinal plants are very rich source of large number of valuable compounds including secondary metabolites which can be used in the treatment of large number of diseases. A number of scientific investigations have highlighted the importance and contribution of many plant families i.e. Asteraceae, Liliaceae, Apocynaceae, Solanaceae, Caesalpinioideae, Rutaceae, Piperaceae and Sapotaceae used as source of medicinal plants. Medicinal plants play a vital role for the development of new drugs, the bioactive extract of these plants should be standardized on the basis of active compounds. Hence, extraction and purification of bioactive compounds in plants has great significance in today's world which involves proper screening of metabolites or compounds obtained from plants.

Extraction of bioactive components from medicinal plants is reported by a number of workers. Srinivasan *et al*. (1995) have studied the kinetics of biomass accumulation and paclitaxel production by *Taxus baccata* cell suspension cultures. Similarly Parc *et al*. (2002) reported production of taxoids by callus cultures from selected *Taxus* genotypes. Wide variety of pharmaceuticals like Taxol from *Taxus* tree, orphine and codeine from Opium poppy, Ginsenosides from *Panax ginseng*, Berberine from *Coptus Japonica*, Diosgenin from *Dioscorea deltoidea*, Camptothecin from *Camptotheca acuminate*,

Vinblastine and Vincristine from *Catharanthus roseus*, Capsaicum from *Capsicum* species are produced through cell culture technique (Vanisree *et al*., 2004).

Extraction of alkaloids from *Tylophora indica* goes back to 1950s when alkaloids were extracted from leaf explants using solvent extraction (Govindachari *et al*., 1954). Alkaloids were isolated from leaves by solvent extraction using methanol, diethyl ether and chloroform. Fresh planting material was grounded with methanol containing 2% acetic acid and was kept in percolator. Combined organic extract was washed with water, dried and concentrated in vacuum to yield crude alkaloidal mass. Characterization of crude extract was done by using TLC. Similar observation for extraction of alkaloid was made by Abe *et al*. (1995).

A process for extracting biologically active alkaloids from *Tylophora indica* and *T. dalzelli* comprising of acid extraction followed by solvent extraction using methanol, ethyl acetate, chloroform was provided by Rao and Brook (US Patent No. 3,497, 593 issued February 24, 1970). Similar studies for isolation of alkaloid tylocrebrine from *Tylophora crebriflora* were carried by Gellert *et al*. (1962). Viswanathan and Pai (1985) reported chemical examination of *Tylophora mollissima* and yielded caffeine as major alkaloid and tylophorine and tylophorinine as minor alkaloids using techniques like ultra violet, infrared and mass spectroscopy.

Ratnagiriswaran and Venkatachalam (1935) had isolated two alkaloids from the plants of *Tylophora asthamatica* (syn. *T. indica*) – tylophorine and tylophorinine which were separated by fractional crystallization of the mixed salts. Similarly Govindachari (2002) isolated tylophorine, tylophorinine, tylophorinidine, septicine and isotylocrebrine by chromatography on alumina and reported the structure of tylophorinine and tylophorinidine with X-ray study.

Chaudhari *et al*. (2005) described an efficient transformation system for *Tylophora indica* using *Agrobacterium rhizogenes* that would result in the rapid and high-frequency induction of transformed roots resulting in higher tylophorine accumulation. Extraction and analysis of tylophorine from powdered roots was observed by de-fatting (0.1DW) roots with petroleum ether for 24h and extracting (by cold percolation) with chloroform.

The final extract obtained after reduction and separation in separatory funnel was analyzed for the presence of tylophorine using preparative HPLC and IR spectra and 1H-neutron magnetic resonance. Root growth and production of tylophorine, the major alkaloid of the plant, varied substantially among the nine root clones studied.

Meera *et al.* (2009) evaluated the diuretic activity from the leaf eaxtracts of *Tylophora indica* from the dried leaf powder with methanol using soxhlet apparatus. Singh *et al*. (2011) gave an overview of chemical composition, pharmacology, toxicology including its antiallergic, antiasthmatic properties in the extracts. Gupta *et al.* (2010) gave an overview of chemical constituents and phytochemical studies of *Tylophora indica*. Mayank *et al*. (2010) developed a method for HPLC fingerprinting analysis for the identification and quantification of marker compound in different extracts of *Tylophora indica*. Reddy *et al.* (2010) investigated the pure and crude extracts of *Tylophora indica* for their antimicrobial and antifeedent activity using 10gm powder of fresh leaves, stem and roots of *Tylophora indica* and extraction in methanol followed by distillation in rotation vapour to get concentrated sample.

Kaur *et al*. (2011) developed a method for mass propagation of *Tylophora indica* from leaf explants and tylophorine was extracted from the leaves of regenerated plants using organic solvents such as hexane, chloforom, and dichloromethane and separated it on high performance thin layer chromatography (HPTLC) using toluene: chloroform: ethanol: ammonia(4:3.5:1.5) as mobile phase. Amount of tylophorine obtained was 80 and 71 µg/ml from callus raised and directly cultured *in vitro* plants respectively.

Material and Methods

Choice of material:

Tylophora indica (Burm.f.) Merr. Commonly known as 'Antmool' or 'Dama Bel', a medicinally important plant belonging to family Asclepiadaceae was selected as an experimental material. This indigenous medicinal plant has multifarious uses and has been used traditionally in the treatment of certain ailments particularly bronchial asthma, bronchitis, allergies, rheumatism and dermatitis. It is a perennial branched climber having cylindrical, twinning stem with long fleshy roots. Leaves are ovate oblong to elliptic oblong and acute at the tip (Fig.1). Flowers are bisexual, minute and grow in umbellate cymes. Corolla is greenish yellow outside and purplish within**.**

Habitat and Distribution:

Tylophora indica is found in the plains, forests, hilly slopes and outskirts of the forests. It forms dense patches in moist and humid conditions and the plant shows stunted growth in the areas with lesser rainfall**.**

Plant is indigenous to India native to the plains and hill forests of Eastern and Southern India upto an altitude of 1260m. It is found in the Sub Himalayan tract from Uttar Pradesh to Meghalaya and in Central and Penninsular India. It also harbors in Ceylon, Malay Island and Borneo**.**

Medicinal Importance:

This plant has been traditionally used as a folk remedy for the treatment of bronchial asthma, bronchitis, whooping cough, dysentery, diarrhoea, inflammations, allergies and in rheumatic gouty pains (Shivpuri *et al.*, 1968. Anonymous 1976).

The leaves and the roots are used medicinally as they have expectorant, diaphoretic and purgative properties. It has reputation as a blood purifier and is regarded as one of the best indigenous substitute for ipecauahna**.**

Fig.1 *Tylophora indica*

Toxic effects:

According to Gupta *et al.* (1979), it may produce some side effects like drowsiness and giddiness. Tightness in throat or chest, chest pain, skin hives, rashes, or itchy or swollen skin may occur in some cases while loss of taste for salt, mouth pain, upset stomach, temporary nausea and vomiting are some other side effects. Preliminary studies showed that the extracts of *Tylophora* are toxic only in extremely high doses while the smaller doses are safe and produce therapeutic effect.

The herb should not be used by the children, pregnant or nursing women or individuals with severe kidney or liver diseases**.**

Active ingredients:

The leaves and roots of this medicinally important plant contain active alkaloids like tylophorine ($C_{24}H_{27}O_4N$), tylophorinine ($C_{23}H_{25}O_4N$), and anti cancerous tylophorinidine ($C_{22}H_{22}O_4N$) which exhibit various pharmacological and biological activities (Bhutani *et al.,* 1984). The major constituent is the alkaloid tylophorine which is responsible for a strong anti-inflammatory action. The other alkaloids include Septicine and Isotylocrebrine. The non-alkaloidal compounds isolated from *Tylophora indica* are kaempferol, quercetin, α- and β- amyrins, tetratriacontanol, octaosanyloctacosanoate, sigmasterol, β-sitosetrol, tyloindane, cetyl-alcohol, wax, resin, coutchone, pigments, tannins, glucose, calcium salts, potassium chloride, quercetin and kaempferol.

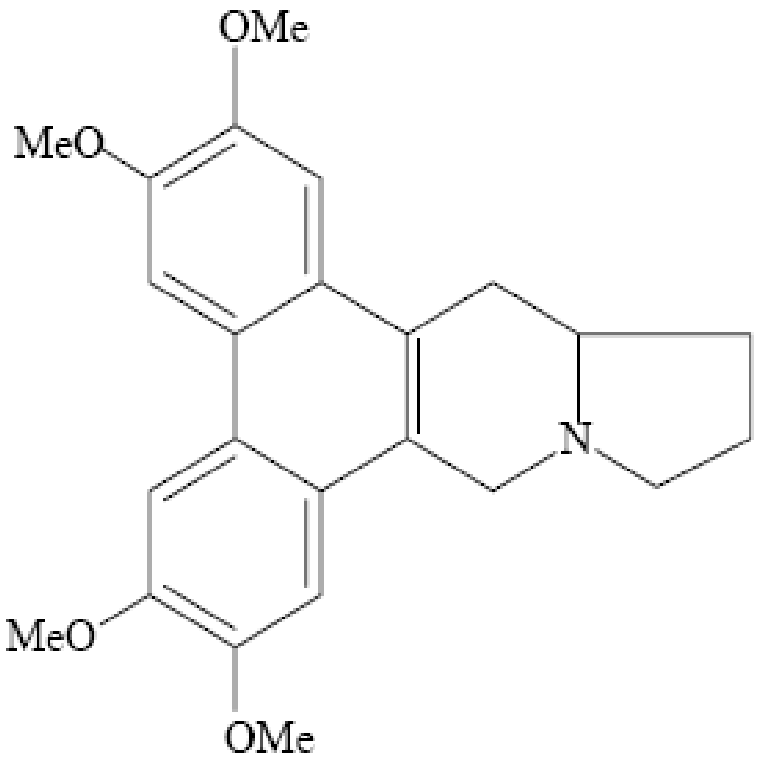
Pharmacology:

In test tube studies, tylophorine is able to interfere with the action of mast cells, which are the key components in the process of inflammation. Hence these results support its traditional use in the treatment of asthma and also its use in anti-allergic medication. According to Bone (1996), the dose should not exceed 200-400mg dried leaf powder per day for the treatment of asthma.

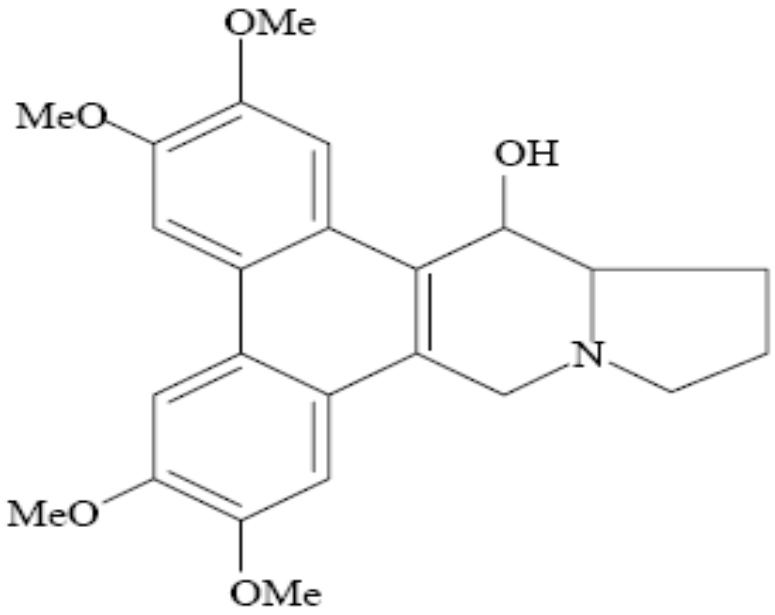
The alcoholic and aqueous extracts of *Tylophora indica* show hepatoprotective activity against ethanol induced hepatotoxicity (Gujrati *et al*., 2007). Crude extracts of *Tylophora*

indica also have been shown to possess antitumour, diuretic and antiallergic activity. Studies with *Tylophora* alkaloids have revealed that they inhibit cellular immune responses and hence show immunomodulatory activity. The major alkaloid tylophorine is responsible for anti-inflammatory activity of this plant (Gopalakrishnan *et al*., 1980**).**

Chemical structure of alkaloids:



Tylophorine



Tylophorinidine

Glassware:

The glassware used for experimental work comprised of conical flasks (100ml, 150ml, 250ml, 500ml, and 1000ml), culture tubes (25× 150mm), and culture bottles (8 × 3inches). In addition other glassware include graduated measuring cylinders, Petridishes, beakers and a range of pipettes (1ml, 2ml, 5ml, and 10ml). Before use all the glassware was subjected to chromic acid solution (mixture of $K_2Cr_4O_7 + H_2SO_4 + H_2O$) followed by thorough washing with tap water. All the vessels were washed with detergent and then cleaned with running tap water and oven dried after rinsing them with distilled water.

Culture media:

Murashige and Skoog's (1962) medium was used as basal medium. Stock solutions of generally 4 times major elements, 100 times minor elements and 10 times organic constituents were prepared. These stock solutions were stored at 4^0C and were mixed in

desired proportions only before use. None of the stock solutions were stored for more than 15 days.

Table1.Composition of Murashige and Skoog's medium (1962)

Ingredients	Concentration(mg/l)
Major elements:	
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$FeSO_4.4H_2O$*	27.8
Na_2EDTA*	37.3
Minor elements:	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	8.3
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic constituents:	
Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
PyridoxineHCl	0.5
Thiamine HCl	0.1
Sucrose	20,000
Agar-agar	10000

*Ferric Na EDTA is the alternative to the use of these two salts and is added freshly to the medium (i.e. 0.04 gm/l)

Definite amounts of all the constituents except agar were mixed and volume was adjusted by distilled water. The pH of solution was adjusted to 5.8± 0.2 using 0.1N NaOH or HCl depending upon high or low. Definite aliquots of medium were distributed depending upon the capacity of culture vessels. Generally 100ml medium was dispersed in each conical flask (250ml), 20ml in each culture tube and 40ml in culture bottles (8×3").

Vessels were plugged with nonabsorbent cotton wrapped in muslin cloth and autoclaved at 15 lbs/in^2 (121^0C) for 15-20 minutes. Test tubes were placed over racks that tilt the test tubes during cooling and gave slanted surface to the agar medium.

Inoculation:

All experimental manipulations were carried out under aseptic conditions in laminar air flow fitted with a bactericidal UV tube (15W, peak emission 2537Å). The floor of chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels and other accessories such as spatula, forceps, needles and scalpel etc., Bunsen burner, match box, tube containing absolute alcohol etc were also cleaned with spirit. Fresh tissue to be inoculated was kept in a Petriplate covered with black paper in order to protect it from harmful Ultraviolet rays. Alcohol was sprayed in the chamber with sprayer. The chamber was then sterilized with Ultraviolet tube kept continuously on for one hour.

Surface sterilization of inoculum:

Just like media, plant tissues were disinfected before inoculation on respective media. Leaf explants were taken from field grown healthy mother plant. These were placed in a bottle covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles and microbes from the surface. The explants were then rinsed with liquid detergent (1% v/v) for another 15 minutes and then washed properly with tap water to remove the detergent. They were treated with bavistin (0.1% w/v) for 5 to 10 minutes to remove the fungal contamination followed by washing with distilled water. Explants were then surface sterilized with 0.1% (w/v) aqueous solution of $HgCl_2$ for 5 minutes followed by 4-5 rinses in sterilized double distilled H_2O in inoculation chamber.

Cultural conditions:

All the cultures were maintained in an air conditioned room at a temperature of 25 ± 4^0C. The source of illumination consisted of 4 feet wide fluorescent tubes (40W) and incandescent bulb (25W). The intensity of illumination was 50µm $m^{-2}s^{-1}$ lux at the level of cultures and 12 hour light regime was followed by 12 hours of darkness.

Extraction of major secondary metabolite – Tylophorine

Extraction of major secondary metabolite was done from leaves of *in vitro* raised plants, callus and suspension cultures.

Leaves were taken from *in vitro* raised field established plant (2 years old), shade dried and grounded to powder by crushing them in pestle and mortar. Weighed amount of powder (50gm) was used for further extractions.

Calli (3-4 month) raised on NK medium was weighed, air dried and grounded to powder using liquid N_2. Weighed amount of powder (20gm) was taken and used for further extraction.

Similarly, suspension cultures were formed by transferring weighed (2gm) amount of callus to liquid MS supplemented with NAA (29.4µM) and Kn (4.65µM). Cultures were kept on shaker for agitation at 120rpm. The fresh and dry weights of cultures were recorded at regular intervals of 5 days till stationary phase was achieved. Cultures were taken at their stationary phase, dried in oven at 40^0C and then grounded to powder. Again weighed amount of powder (20gm) was taken for further extraction

Procedure: Extraction

50gm powder of leaves and 20 gm powder of callus and suspension cultures was soaked in 40ml of chloroform and mixture was stirred for 2-3 hours using soxhlet, followed by filtration. The filtrate was then reduced on rotary flash evaporator at 50^0C. The resultant extract was extracted using 40ml of ethyl acetate and HCl (0.5N) in the ratio of 1:1 in a separatory funnel. Two separate layers were formed. Lower layer of HCl was collected and pH was adjusted between 8-9. The process was repeated twice and every time lower layer was collected. This extracted layer was reduced using flash evaporator and to this pooled exract, methanol was added and kept for further analysis.

Purification of secondary metabolite

Purification was achieved by thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC).

For TLC, Silica gel was coated on glass plates in organic solvent methanol using TLC applicator. Application of the sample was done by using fine capillary. It was kept in the developing chamber saturated with developing solvents comprising of Toluene: Ethyl acetate: diethyl amine in different ratios 7:2:1, 5:3:1, and 6:2:2. Optimization of the solvents was done to find out best solvent ratio. The chamber was covered with glass plate and left undisturbed for some time. When the solvent has moved to 75% of the solvent front on the glass plate, it was taken out and kept in iodine chamber for the development of spots.

The solvent system that gave the best results on TLC was then used in HPTLC for the fine purification and detection of tylophorine. Plant extract was applied with 100 µl syringe on pre coated silica gel 60F254 HPTLC plates (10 x 10 cm) with band length of 6 mm and track separation of 12 mm using Linomat V applicating device and nitrogen as spray agent. The chromatograph was developed in twin trough chamber using the same solvent system as for TLC and scanned in scanner III at 258 nm wavelength using lamp in absorption mode with spectrum scan speed of 100 nm/s. R_f value was calculated and compared with R_f value (0.64) of standard tylophorine obtained from library spectra.

Observations and results

Leaf segments were excised from field grown mature plant of *Tylophora indica* and were used for the direct induction of adventitious shoots and callus formation. Leaf explants (4-5mm in size) were cultured on MS medium supplemented with different growth regulators used either alone or in conjunction with each other.

De novo adventitious shoot induction

Murashige and Skoog's (MS) medium supplemented with various concentrations and combinations of cytokinins (Kn, BAP) and auxins (NAA, IAA, IBA, 2, 4-D) was used for the regeneration of multiple adventitious shoots from the leaf explants. Twenty replicates were used for each treatment and cultures were subcultured after every 7-8 weeks.

Nodular meristemoids differentiated from the cut ends and from abaxial and adaxial surface of leaf lamina when cultured on BAP (8.8μM) supplemented medium. These nodular meristemoids covered the entire surface of the explant with in 2-3 weeks (fig.2). Eventually these meristemoids developed into green leafy shoots in about 80% of cultures (figures 3 & 4). The shoots elongated and developed many leaves (fig.5). Nearly 25 shoots were formed per culture after 8 weeks of culturing.

Leaf explants cultured on MS medium supplemented with 2, 4-D (9.74μM) and BAP (4.4μM) also induced the formation of nodular meristemoids but the number of shoots formed were less and sprouting of shoot buds took longer time of 7 weeks.

Murashige and Skoog's medium supplemented with BAP (22μM) and adenine sulphate (1.35μM) produced the greatest number of shoot buds directly from the leaf explants. Nodular meristemoids differentiated from the entire cut surface of leam lamina after 8-10 days of culturing and within 3 weeks entire surface was covered with these meristemoids Eventually these meristemoids grew into leafy shoots after 5 weeks in 90% of cultures(fig.6). Repeated subculturing accelerated the formation of shoots in large numbers (50-60 per culture) without any decline in proliferation (fig.7). Effect of various concentrations of BAP and adenine sulphate on direct shoot regeneration after 8 weeks is depicted in Figure. 8.

Rooting of Microshoots

Regenerated microshoots were carefully rescued from the flasks and were inoculated upright in MS medium with or without growth hormones. Shoots were inoculated on half strength and full strength BMS medium and MS medium supplemented with different auxins like IBA, IAA, and NAA for root initiation. Among the various auxins tested, IBA (9.8µM) showed good results where roots initiated after 15 days. Best root initiation, however, occurred on half strength MS medium where healthy roots emerged in 90% of the cultures. The roots were long and white having root hairs as well (fig.9). Complete plantlets with elongated shoot and root systems were formed after 30 days (fig.10).

Accilimatization and transfer of plantlets to the soil

The rooted plantlets were successfully transferred to the field conditions through successive hardening stages. The rooted plantlets were gently removed from the cultures tubes keeping the roots intact by using forceps with extreme care to avoid any mechanical damage to the plantlets. Roots were thoroughly washed with tap water to remove any remaining agar sticking to them. Firstly the rooted plantlets were acclimatized on moist cotton for 10-12 days (fig.11) followed by their transfer to plastic cup containing sterile potting mixture of soil and vermicompost in the ratio of 1:1 under the high relative humidity in the growth room (fig.12). The plants with newly formed leaves were shifted to green house for 2 weeks and eventually established in the soil (fig.13). Nearly 90% of the plants survived with no phenotypic variations observed among the regenerated plants when compared to the parent plant.

Callus induction

Callusing of the leaf segments occurred on MS medium supplemented with different concentrations of NAA either alone or in conjunction with Kn or BA. Syngergistic action of NAA (29.4µM) with Kn (4.65µM), hereby, designated as NK medium was most effective in the initiation and sustained growth of the calli. Callusing occurred either at the cut ends or along the entire surface of the leaf segment after 8-10 days of culturing and within 3 weeks entire segment turned into a mass of green and compact callus

(fig.14). Callus when subcultured on NK medium proliferated further and showed sustained growth (fig.15). Leaf explants also showed callus induction on MS medium supplemented with 2,4-D (38.96µM)+ Kn (4.65µM), but growth of callus was slow and it took nearly 45 days for the explants to turn into mass of callus.

Extraction and purification of major secondary metabolite- Tylphorine

Extraction of the major secondary metabolite tylophorine was carried out from the dried leaf powder of *in vitro* raised field established plant (2 years old), calli (3-4 months old) formed from the leaf explants on NK medium and dried suspension cultures selected after interval of 25 days at stationary phase. In stationary phase, accumulation of secondary metabolites is maximum. Hence, suspension cultures at stationary phase were used for the extraction and purification of tylophorine.

Regular monitoring of suspension cultures with regard to the growth was done after specific intervals and onset of stationary phase was observed on the 25^{th} day. Growth curve was plotted in terms of fresh and dry weights of the culture (Fig16.). Lag phase was observed for the first 5 days followed by the log phase which started after 5^{th} day and lasted till 24^{th} day. On day 25, stationary phase was observed till day 28 after which decline in both fresh and dry weights was observed indicating decline in the growth (table 2). Hence, cell cultures were selected from 25^{th}-28^{th} days during the stationary phase, oven dried at 40^0C and made into fine powder.

Powdered *Tylophora* samples from all the above mentioned sources were extracted with chloroform using soxhlet apparatus followed by extraction with ethyl acetate and HCl in separatory funnel. The extracted sample was reduced under vacuum and was suspended in methanol. The methanolic mother liquor exhibited different components on thin layer chromatography in solvent system of different ratios (7:2:1, 5:3:1, and 6:2:2). The best results, however, were observed using toluene: ethyl acetate: diethyl amine in the ratio of 7:2:1. The spots were visualized using iodine vapors. Isolated tylophorine was further purified, analyzed and characterized using high performance thin layer chromatography (HPTLC).

HPTLC analysis

HPTLC was carried out by using the same solvent system as optimized in thin layer chromatography. The mother liquor was loaded on pre coated silica gel 60F254 HPTLC plates (10×10 cm plates) using 100µl syringe. Track 1 contains dried leaf powder whereas track 2 and 3 contains dried callus and suspension powders respectively (Figures17, 18 &19). Plates were developed in the solvent system of toluene: ethyl acetate: diethylamine (7:2:1) and scanned at 258 nm. The mother extract exhibited various components but only tylophorine could be identified on comparison of R_f values with the standard from library spectra. Track 1 showed 8 different peaks with 7^{th} peak corresponding to standard tylophorine having an R_f value of 0.63 and 5.29% peak area. Track 2 shows 10 different peaks with 8^{th} peak corresponding to standard at an R_f value of 0.64 and 6.62% peak area. In track 3, peak 7 showed R_f value of 0.64 comparable with the standard. The percentage area of peak was 7.45 (Table 3). R_f value of the peaks in all the three samples was comparable to that of standard which confirmed the presence of tylophorine in the given samples. 3-D display of all the peaks at 258nm is also shown (Fig.20). HPTLC analysis showed that among different combinations of solvent system tried toluene: ethyl acetate: diethylamine (7:2:1) showed optimum percentage of tylophorine in all the three samples.

Figure 2. Nodular meristemoids covering the entire surface of leaf explants on MS + BAP (8.8μM) after 15 days of culturing.

Figures 3 & 4. Sprouting of shoots from meristemoids after 4 and 6 weeks on MS + BAP (8.8μM).

Figure 5. A well developed shoot having many leaves after 8 weeks.

Fig.2

Fig.3

Fig.4

Fig. 5

Fig.6 Meristemoids growing into leafy shoots after 5 weeks of culturing on MS + BAP (22µM) + Adenine sulphate (1.35µM).

Fig. 7 Formation of large number of shoots on MS supplemented with BAP (22µM) + Adenine sulphate (1.35µM) after 8 weeks.

Fig.8. Histogram showing effect of different concentrations of BA and adenine sulphate on direct shoot regeneration from leaf explant.

Fig.6

Fig.7

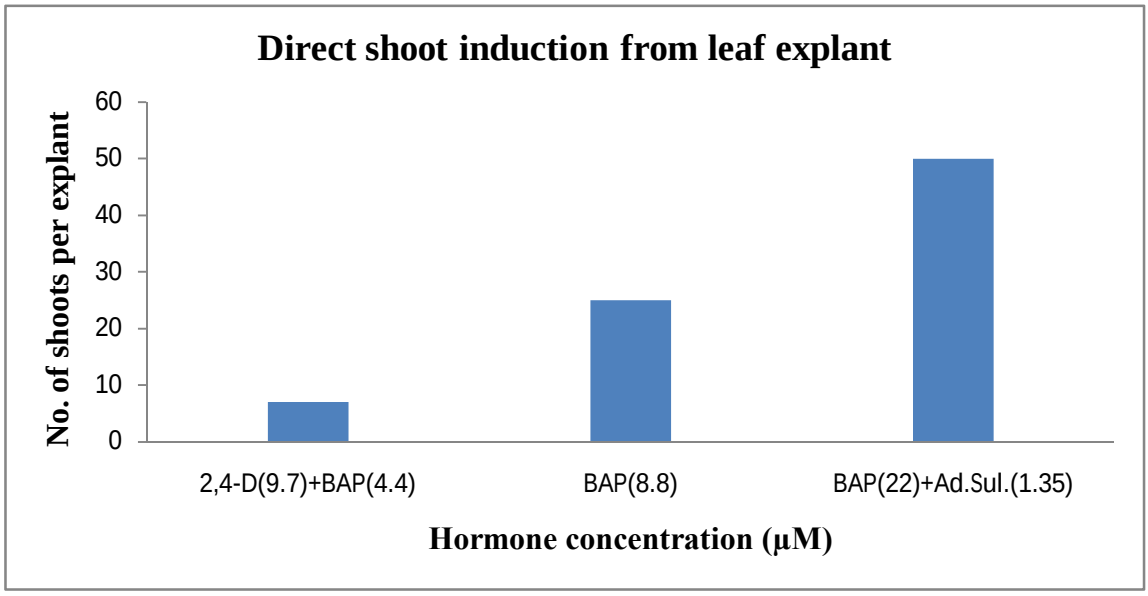


Fig.8

Fig. 9 Rooting from the basal end of regenerated shoot on half strengh BMS medium after 15 days of planting.

Fig.10 A complete plantlet with well developed root and shoot system formed after 30 days.

Fig.11 The rooted plantlet acclimatizing on moist cotton.

Fig.12 Plantlet transferred to plastic pot containing soil and vermicompost in the ratio of 1:1.

Fig.13 A 4-month- old well acclimatized plant in the soil.

Fig 9

Fig.10

Fig.11

Fig.12

Fig.13

Fig .14 A 3 week-old leaf culture showing callusing on NK medium.

Fig. 15 A 4- weeks-old leaf callus on NK medium showing sustained growth.

.

Fig. 14

Fig.15

Fig. 16 Growth curve of suspension culture on liquid NK medium.

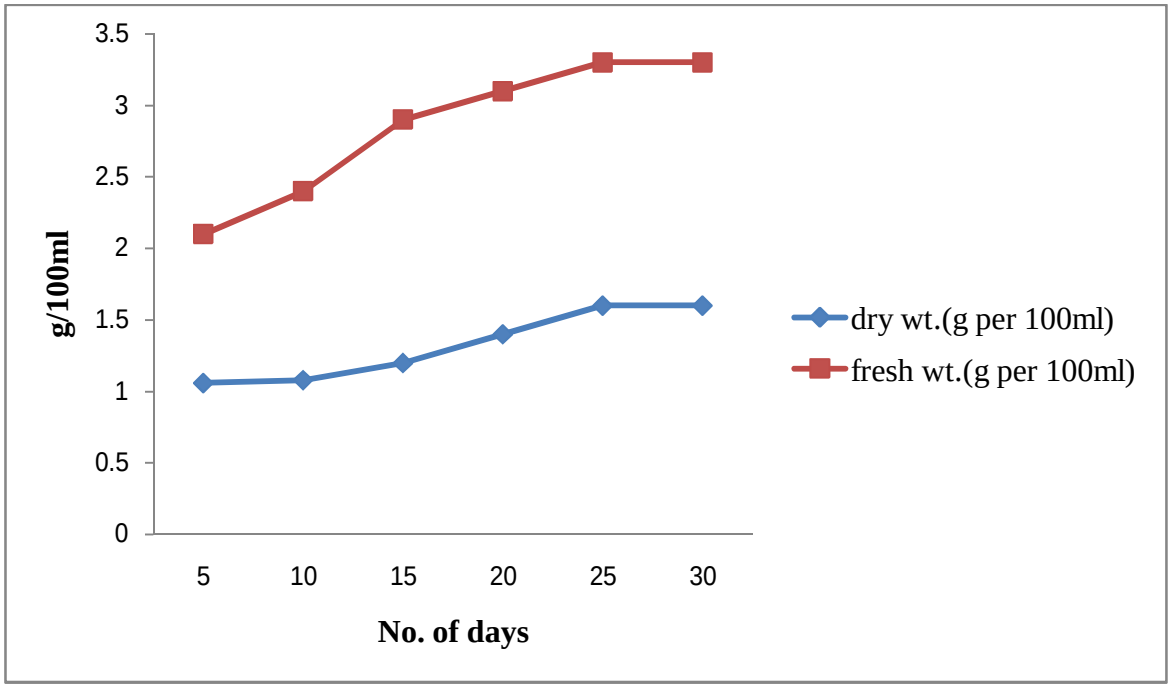


Fig.16

Table.2 Fresh and dry weights of suspension culture after interval of 5 days

No. of days	Fresh wt.(g per 100ml)	Dry wt.(g per 100ml)
5	2.1	1.06
10	2.4	1.08
15	2.9	1.2
20	3.1	1.4
25	3.3	1.6
30	3.3	1.6

Figures 17, 18 & 19 HPTLC of crude extract of leaves of *in vitro* raised plant, dried callus and suspension powders respectively showing different peaks including the peak for the standard at 258 nm.

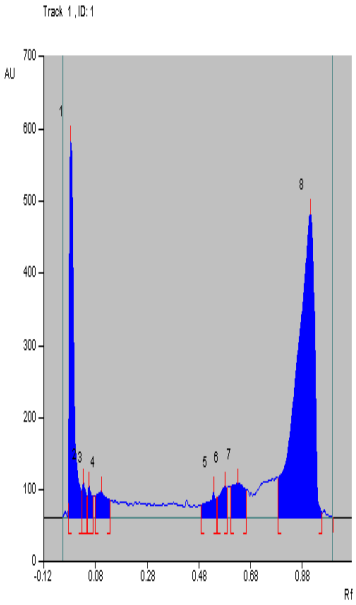


Fig. 17

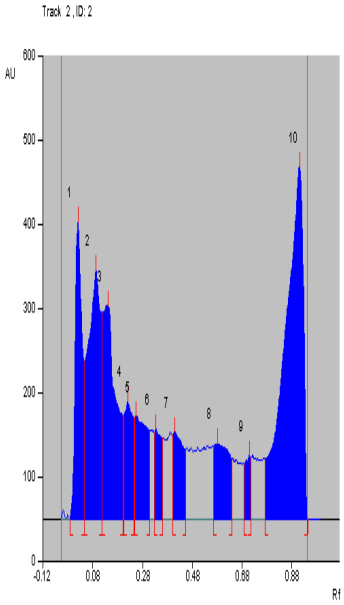


Fig.18

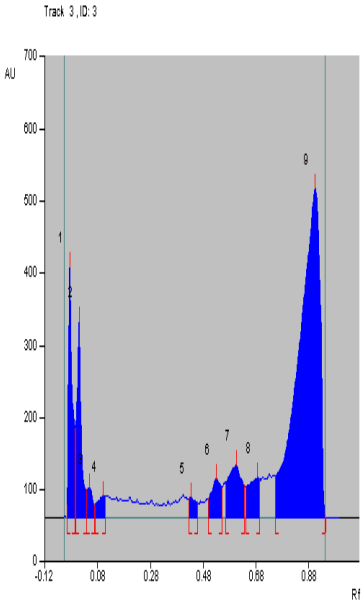


Fig.19

Table.3 Showing presence of tylophorine in all the samples based on R_f values when compared to the standard.

Track	Peak	(R_f)	Area (AU)	Area %	Sample Identification
1	7	0.63	1826.6	5.29	Standard
2	8	0.64	5192.5	6.62	Standard
3	7	0.64	3616.9	7.45	Standard

Figures.20 A 3-D display of all the tracks at 258 nm.

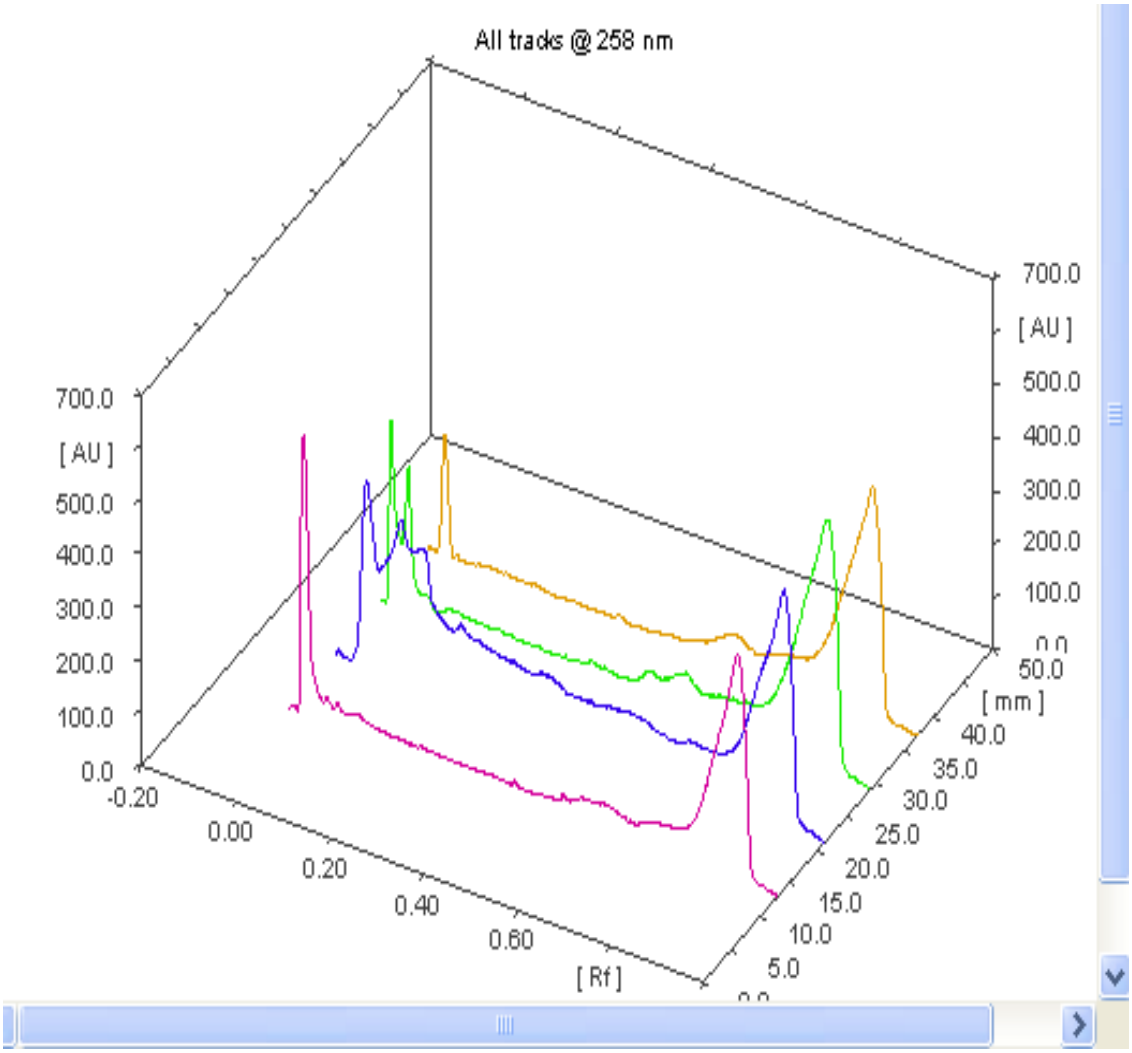

All tracks @ 258 nm
700.0
[AU]
500.0
400.0
300.0
200.0
100.0
0.0
-0.20
0.00
0.20
0.40
0.60
[Rf]
700.0
[AU]
500.0
400.0
300.0
200.0
100.0
50.0
[mm]
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0

Fig.20

Discussion

The present investigation was undertaken on an important medicinal plant *Tylophora indica* with a view to develop an efficient, reliable and reproducible protocol for its clonal propagation under *in vitro* conditions and to extract major secondary metabolite – tylophorine from *in vitro* raised plants, callus and suspension cultures.

Tylophora indica is a threatened medicinal plant having multifarious uses. Due to the lack of organized cultivation and over exploitation, the wild population of this plant is declining very fast. It is now listed as one of the plant species in India vulnerable to extinction. Therefore, it is necessary to develop an efficient protocol for large scale multiplication of this endangered species.

Tylophora has great organogenic potential as it exhibited prolific shoot bud formation and plant regeneration from leaf segments under *in vitro* conditions. Direct organogenesis is regarded as the most reliable method for clonal propagation because it upholds genetic uniformity among progenies, unlike those regenerated from callus tissue. For shoot proliferation, Cytokinins are one of the most important factors affecting the response (Lane 1979, Stolz 1979, Bhojwani 1980, Garland and Stolz 1981). A wide range of cytokinins like Kinetin, BAP, 2-ip and Zeatin has been employed in shoot proliferation (Bhojwani and Razdan 1983). A number of plants such as Blueberries (Cohen 1980), Garlic (Bhojwani, 1980), Annatto (Sharon and D'Souza, 2000) and *Gardenia jasminoides* (Chuenboonngarm, 2001) have been successfully multiplied by 2-ip. However, a wide survey of literature suggests that BAP is the most reliable and effective cytokinin.

In the present study, *de novo* adventitious shoot formation occurred on MS medium supplemented with different concentrations of BAP alone or in conjunction with adenine sulphate. However the best results were observed on MS medium supplemented with BAP (22µM) & adenine sulphate (1.35µM) where optimum growth of meristemoids occurred from the leaf segments. These meristemoids when cut into small groups and subcultured on fresh inductive medium multiplied further resulting in an exponential

increase in number and eventually developed into green leafy shoots in nearly 85% of the cultures. Chaudhari *et al.* (2004) reported the formation of organogenic nodular meristemoids from root explants on MS medium supplemented with BAP (10.77-26.8µM) which subsequently developed into shoot buds in 42% of the cultures. Similarly, Nema *et al*. (2007) reported the formation of nodular callus from leaf explants of *Tylophora indica* on MS medium supplemented with BAP (11µM) and shoot formation on MS + BAP (11µM) + IAA (0.56µM). Our results are in accordance with the results reported by Bera & Roy (1993) who reported multiple shoot formation directly from leaf explants of *Tylophora indica* on MS supplemented with BAP (5mg/l) and adenine sulphate (0.5mg/l).

Induction and development of roots at the base of regenerated shoots is an essential and indispensible step for establishing these plantlets in the soil. The shoots thus formed were excised and transferred for rooting on half strength MS and MS medium supplemented with different concentrations of IAA, IBA and NAA. Amongst the various auxins used, IBA proved to be the best. However, excellent root induction occurred on half strength MS medium alone. Similarly, Thomas and Philip (2005) and Faisal and Anis (2005 a) have reported IBA to be optimal for rooting in the regenerated shoots of *Tylophora indica*. However, Bera and Roy (1993) reported IAA to be the best rooting medium. The rooted plants were first hardened under *in vitro* and green house conditions for 4 weeks before final transfer to the field. During the period of hardening, proliferation of roots and emergence of new leaves was observed and there was substantial improvement in their survival rate which was nearly 90%. The present study demonstrates production of large number of uniform clones for commercial production and germplasm conservation of *Tylophora indica*.

For callus induction from leaf explants, MS medium was supplemented with different growth hormones. Synergistic combination of NAA (29.4µM) + Kn (4.65µM) , hereby designated as NK medium, gave the best results leading to the formation of green and solid callus showing sustained growth. Callusing also occurred on MS medium supplemented with 2, 4-D (38.96µM) + Kinetin (4.65µM), but the growth of callus was slow. Jayanti and Mandal. (2001) reported callusing from leaf explants of *Tylophora*

indica on MS medium with 2, 4-D (9.78µM) and Kinetin (4.65µM). Faisal and Anis (2005 b) reported callus induction from stem and petiole segments of *Tylophora indica* on MS medium supplemented with 2, 4, 5-T (10µM) and 2, 4-D and TDZ respectively. Thomas and Philip (2005) achieved callus formation from mature leaf pieces on MS supplemented with 2, 4-D (7µM) and BAP (1.5µM) in 92% explants.

Suspension cultures were prepared by transferring callus to liquid MS medium supplemented with NAA (29.4µM) and Kn (4.65µM). Cultures were incubated at $25^0 \pm 4$ C at 120 rpm. After regular interval of 5 days, suspension cultures were harvested by taking their fresh and dry weights. Growth curve was plotted depicting lag phase for first five days, followed by log phase till 24^{th} day and then stationary phase during 25^{th}-28^{th} day. At stationary phase, suspension cultures were dried at 40^0C, grounded to powder. Similarly leaves and calli were shade dried and grounded to powder. Powdered leaves, calli and suspension cultures were then used for further extraction of major secondary metabolite - tylophorine. First, TLC was performed by using different combinations of the solvents like toluene: ethyl acetate: diethyl amine but best results were observed on 7:2:1. Then further fine purification of the sample was done by performing high performance thin layer chromatography (HPTLC). Presence of tylophorine was confirmed in all the samples based on retention factor (R_f) values compared with standard when HPTLC plates were developed in solvent system comprising of toluene: ethyl acetate : diethylamine (7: 2:1) and scanned at 258nm.

Earlier alkaloid productivity was studied in stem and root callus cultures of *Tylophora indica* by Benzamine and Mulchandani, 1973 and by some other workers, but they failed to detect these alkaloids. Presence and purification of tylophorine and other alkaloids was reported using methods of Infrared spectroscopy (Rao *et al*., 1970) or HPLC (Chaudhari *et al.,* 2005 and Jha *et al.,* 2005). However not much has been reported on the detection of tylophorine from regenerated plants using HPTLC techniques. Jha *et al*., 2005 and Chaudhary *et al*., 2005 using HPLC technique revealed the presence of tylophorine from the root regenerated plants of *Tylophora indica* but the amount of tylophorine was lower than field plants. However, they reported tylophorine content of transformed root clones

1.2-1.5 times higher than the roots of non wild plantlets, which was attributed to the genetic transformation induced autonomous growth of transformed roots.

Kaur *et al*. (2011) extracted tylophorine from the leaves of regenerated *Tylophora* plants using organic solvents such as hexane, chloroform and dichloromethane and separated on HPTLC using toluene: chloroform: ethanol: ammonia (4:3:5:1:5) as mobile phase.

The present study demonstrates the high regenerative efficiency of *Tylophora indica* for rapid and mass scale propagation with potential for the production of secondary metabolites under *in vitro* conditions.

Literature cited

- Abe, I., Yukiko, Yamauchi, T., Honda, K. and Hayashi, N. 1995. Phenanthroindolizidine alkaloids from *Tylophora tanakae*. Phytochemistry., 39:695–699.

- Annapurna, D. and Rathore, T.S. 2010. Direct adventitious shoot induction and plant regeneration of *Embelia ribes* Burm F. Plant Cell, Tissue and Organ Culture (PCTOC) Journal of Plant Biotechnology. 101:269-277.

- Anonymous. 1976. The Wealth of India. Raw materials., 10: Publications and Information Directorate. CSIR, New Delhi, India.

- Attisso, M.A. 1983. Phytopharmacology and Phytotherapy, in Bannerman, R.H., Burton, J., and Ch'en Wen- Chieh (eds.), Traditional Medicine and Health Care Coverage. World Health Organization, Geneva.

- Baskaran, P. and Jayabalan, N. 2010. Direct organogenesis from hypocotyle explants of *Psoralea corylifolia* L.- an endangered medicinal plant. Indian Journal of Biotechnology., 9: 329-332.

- Beegum, Shahanaz A., Martin, K.p., Zhang, chun-lai., Nishita, I.K., Ligimol., Slater, Adrian. and Madhusoodan, P.V. 2006. Organogenesis from leaf and internode explants of *Ophiorrhiza prostrate*, an anticancer drug (Camptothecin) producing plant. *Elect. Journal of Biotechnolgy* ISSN: 0717-3458., 10: 1-10.

- Benzamine, B. D. and Mulchandani, N. B. 1973. Studies in biosynthesis of secondary constituents in tissue cultures of *Tylophora indica.* Planta Medica., 23: 394-397.

- Bera, T.K. and Roy S. C. 1993. Micropropagation of *Tylophora indica* by multiple shoot formation from mature leaf explants without callus intervention Bot. Bull. Acad., 34: 83-87.

- Bhojwani S.S .1980. *In vitro* propagation of garlic by shoot proliferation. Sci. Hortic., 13:47-52.

- Bhojwani, S.S. and Razdan, M.K. 1983. Plant Tissue Culture: Theory & Practice, Elsivier Sci. Publ. Amsterdam: 1-50.

- Bhutani, K. K. and Atal, C. K. 1984. Alkaloids from *Tylophora hirsuits*" Phytochemistry., 23 (8) :1765-1769.

- Bone K., 1996. Clinical Applications of Ayurvedic and Chinese Herbs. Warwick, Queensland, Australia: Phytotherapy Press., 134-6.

- Bourgaud, F., A, Gravot., Miles, S. and Gonthier, E. 2001. Production of plant secondary metabolites: a historical perspective. Plant Sciences.161:839-51.

- Chaudhari, K. N., Ghosh, B., Tepler, D. and Jha, S. 2005.Genetic transformation of Tylophora indica with agrobacterium rhizogenes A4: growth and tylophorine productivity in different transformed root clones. Plant cell reports., 24: 25-35.

- Chaudhari, K. N., Ghosh,, B. and Jha, S. 2004. The root: a potential new source of competent cells for high frequency regeneration in *Tylophora indica*. Plant Cell reports., 22:73-740.

- Chuenboonngarm N., Suvimon C. and Bhamarapravati S. 2001. Effect of BA and 2-iP on shoot proliferation and somaclonal variation of *Gardenia jasminoides* Ellis *in vitro* culture. Science Asia., 27: 137-141.

- Cohen, D. 1980. Application of micropropagation methods for blueberries and tamarillos. Comb. Proc. IntI. Plant Prop. Soc., 30:144-146.

- Faisal, M. and Anis, M. 2005a. An efficient *in vitro* method for mass propagation of *Tylophora indica*" Biologia Plantarum., 49:257-260.

- Faisal, M. and Anis, M. 2005b. *In vitro* regeneration and plant establishment of Tylophora indica: Petiole callus culture. *In vitro* Cellular and Development Biology, Plant., 41: 511-515.

- Farnsworth, N.R. and D.D. Soejarto. 1991. Global Importance of Medicinal Plants. In Conservation of medicinal plants. Akarele, A. *et al.* (eds).

- Garland, P., Stolz, L.P .1981. Micropropagation of Pissrdi plum, Ann. Bot. vol., 48:387-389.

- Gellert, E.T.R., Govindachari M.V., Lakshmikantham, I.S., Ragade, R., Rudzals, N. and Viswanathan J. 1962. The alkaloids of *Tylophora crebriflora*: structure and synthesis of tylocrebrine, a new phenanthroindolizidine alkaloid. J. Chem. Scom: 1008-1014.

- Gopalakrishnan, C., Shankaranarayanan, D., Nazimudeen, S.K., and Kameswaran, L.1980. Effect of tylophorine , a major alkaloid of *Tylophora indica*, on immunological and inflammatoryreactions. Indian J. Med. Res., 71: 940–948.

- Govindachari, T.R .2002. Five decades in the study of natural products. Proc. Indian Acad. Sci., 3: 114.

- Govindachari, T.R., Pai, B.R. and Nagarajan, K. 1954. Chemical Examination of *Tylophora asthmatica*. Part I. J. Chem. Soc*:* 2801–2803.

- Gujrati, V., Patel, N., N, Rao., Venkat., Nandakumar, K., Gouda, T.S., Shalam, Md., Kumar and Shanta S.M. 2007. Hepatoprotective activity of alholic and aqueous extracts of leaves of *Tylophora indica*. (Linn.) in rats. Indian J Pharmacol., 39:43-47.

- Gupta, M., Mukhtar, H.H. and Ahmad, Sayeed. 2011. Phytopharmacological and plant tissue culture overview of *Tylophora indica* (burm f.) Merill. J. Pharm. Sci. & Res., 2 (7).

- Gupta, S., George, P., Gupta, V., Tandon, R. and Sundaram, K.R. 1979. *Tylophora indica* in bronchial asthma- a double blind study. Ind J Med Res., 69: 981-989.

- Gurib- Fakim, A., 2006. Review-Medicinal plants: Traditions of yesterday and drug of tomorrow. Mol. Aspects Med., 27: 1-93.

- Hedayat, M., Abdi. Gh. and Khosh-Khui, M. 2009. Regeneration via Direct Organogesis from leaf and petiole segments of Pyrethrum (*Tanacetum cinerariifolium)*(Trevir.) Schultz-Bip. American-Eursian. J. Agric. & Environ. Sci., 6(1): 81-87.

- Jayanti, M. and Mandal, P.K. 2001. Plant regeneration through somatic embryogenesis and RAPD analysis of regenerated plants in *Tylophora indica* (Burm. F. Merill)" *In vitro* Cell. Dev. Biol.-Plant., 37:576-580.

- Jha, Sumita., Bandyopadhya, M., Chaudhary, K. N., Ghosh, S and Ghosh, B. 2005. Biotechnological approaches for the production of forskolin , withanolides, colchicines and tylophorine. Plant genetic resources., 3(2):101-115.

- Kaur, Harmanjit., Anand, Manju. and Goyal, Dinesh. 2011, Extraction of tylophorine from in vitro raised plants of *Tylophora indica.* Journal of Medicinal Plants Research., 5(5): 729-734.

- Kulkarni, Anjali., Thengane, S.R. and Krishnamurthy, K.V. 2000. Direct shoot regeneration from node , internode, hypocotyl & embryo explants of *Withania somnifera*. Plant cell, Tissue & Organ culture., 62: 203-209.

- Lane W.D. 1979. *In vitro* propagation *Spirea bumalda* and *Prunus cistena* from shoot apices. Canadian Journal of Plant Science., 59: 1025-1029.

- Mayank, G., Mhaveer, S., Mukhtar Hayat, M. and Sayeed A. 2010. HPTLC fingerprinting of different leaf extracts of *Tylophora indica* (Burm f.) Merill. Pharmacognosy Journal., 2(11): 381-385.

- Meera, R., Devi, P., Muthumani, P., B, Kameshawari. And P. Eswarapriya. 2009. Evaluation of Diuretic activity from *Tylophora indica* leaves extract. J. Pharma science & Research., 1(3): 112-116.

- Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant., 15: 473- 497.

- Nema, RK., Ramawat, KG., Gupta, Gd., Tanwar, YS. and Mathur, Meeta. 2007. Rapid micropropagation of *Tylophora indica*. Pharmacognosy., 3(9): 52-55.

- Parc, G., Canaquier, A., Landre P., Hocquimiller, R., Chriqui, D. and Meyer, M. 2002. Production of taxoids with biological activity by plants callus culture from selected *Taxus* genotypes. Phytochemistry., 59 (7): 725-30.

- Pei, S.J., 2001. Ethnobotanical approaches of traditional medicine studies some experiences form Asia, Pharma Bio., 39: 74-79.

- Rao, K. V., Brook, Pine. and N.J. 1970. Alkaloids of *Tylophora indica* and *Tylophora dalzelli"* US Patent No.3, 497,593.

- Ratnagiriswaran A.N. and Venkatchalam K .1935. The chemical examination of *Tylophora asthmatica* and isolation of alkaloids tylophorine and tylophorinine. Indian J. Med. Res., 22 (3): 433-441.

- Reddy, B. Krishna., Balaji, M., Reddy, P. Uma., Sailaja, G., Vaidyanath, K. and Narasimha, G. 2009. Antifeedent and antimicrobial activity of *Tylophora indica*. African Journal of Biochemistry Research., 3(12), 393-39.

- Sharon, M. and D'Souza, M.C .2000. *In vitro* clonal propagation of annatto (Bixa orellana L.). Current Science., 78(12): 1532-1535.

- Shivpuri, D.N., Singhal, S.C. and Prakash, D. 1968. Preliminary studies in Tylophora indica in the treatment of asthma and allergic rhinitis. J. Ass. Phys. India., 15(1): 9–15.

- Siddhiqui, M.A.A., John, A.Q. and Paul, T.M. 1995. Status of some important medicinal and aromatic plants of Kashmir Himalaya. Advances in Plant Sciences., 8:134-139.

- Siddique, I., Anis, M. and Aref, I.M. 2010. *In vitro* adventitious shoot regeneration via indirect organogenesis from petiole explants of *Cassia*

angustifolia Vahl,-a potential medicinal plant. Appl Biochem Biotechnol., 162(7): 2067-74.

- Singh, A., Duggal, S. and Katekhaye, Sabkar. 2011. *Tylophora asthmatica* Wight and Arn. - Review NPT., 1(1):1-4. ISSN: 2231-0436.

- Srinivasan, V., Pestchanker, Luis., Moser, S., J. Hirasuna, Thomas., A. Taticek, Ronald. and L. Schuler, Micheal. 1995. Taxol production in bioreactors: Kinetics of biomass accumulation, nutrient uptake, and taxol production by cell suspensions of *Taxus baccata*. Biotechnology & Bioengineering., 47: 666-676.

- Srivastava, J., J. Lambert and N. Vietmeyer. 1995. Medicinal Plants: A Growing Role In Development. World Bank, Washington D.C.

- Stolz LP. 1979. *In vitro* propagation of Acalypha wilkesiana. HotSci., 80:290-292.

- Tempesta, M. S. and S. King. 1994. Tropical Plants as a Source of New Pharmaceuticals. In P.S. Barnacal (ed.) Pharmaceutical Manufacturing International: The International Review of Pharmaceutical Technology Research and Development. Sterling Publications Ltd., London.

- Tewari, D. N. 1996. Economic and Ecological Rehabilitation of Himalaya Through Sustainable Commercialization of Medicinal Plants Resources. Paper presented at INBARIIDRC/IPGRI/ICIMOD Workshop on the Role of Medicinal Pants, Bamboo and Rattan in Mountain Development. IDRC, New Delhi.

- Thomas, D. and Philip, B. 2005. Thidiazuron-induced high frequency shoot organogenesis from leaf- dried callus of a medicinal climber, *Tylophora indica*. *In vitro* cellular and Development Biology- Plant., 4:124-128**.**

- Vanisree, Mulabagal., Lee, Chen-Yue., Lo, Shu-Fung. and Nalawade, S.M., Lin. 2004. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. Bot. Bull. Acad. Sin., 45: 1-22.

- Ved, DK. and Ravi kumar, K. 2000. 100 Red Listed Medicinal Plants of Conservation Concern in South India, FRLHT Publications, 92-100.

- Viswanathan, N. and Pai B.R .1985. Alkaloids of *Tylophora mollisima* J. Nat. Prod., 48(6): 997-998.